Friend turned foe: selfish behavior of a spontaneously arising mitochondrial deletion in an experimentally evolved Caenorhabditis elegans population

Abigail N. Sequeira,1,2 Ian P. O’Keefe,1,3 Vaishali Katju1,4,* Ulfar Bergthorsson1,4,*

1Department of Veterinary Integrative Biosciences, Texas A&M University, 402 Raymond Stotzer Parkway, College Station, TX 77845, USA
2Department of Biology, Pennsylvania State University, 208 Mueller Laboratory, University Park, PA 16802, USA
3Department of Biochemistry and Molecular Biology, University of Maryland, 655 W. Baltimore Street, Baltimore, MD 21201, USA
4Program in Evolutionary Biology, Department of Ecology and Genetics (IEG), Evolutionsbiologiskt centrum, Norbyvägen 18D, Uppsala University, 752 36 Uppsala, Sweden

Selfish mitochondrial DNA (mtDNA) mutations are variants that can proliferate within cells and enjoy a replication or transmission bias without fitness benefits for the host. mtDNA deletions in Caenorhabditis elegans can reach high heteroplasmic frequencies despite significantly reducing fitness, illustrating how new mtDNA variants can give rise to genetic conflict between different levels of selection and between the nuclear and mitochondrial genomes. During a mutation accumulation experiment in C. elegans, a 1,034-bp deletion originated spontaneously and reached an 81.7% frequency within an experimental evolution line. This heteroplasmic mtDNA deletion, designated as meuDf1, eliminated portions of 2 protein-coding genes (coxII and nd4) and tRNA-thr in entirety. mtDNA copy number in meuDf1 heteroplasmic individuals was 35% higher than in individuals with wild-type mitochondria. After backcrossing into a common genetic background, the meuDf1 mitotype was associated with reduction in several fitness traits and independent competition experiments found a 40% reduction in composite fitness. Experiments that relaxed individual selection by single individual bottlenecks demonstrated that the deletion-bearing mtDNA possessed a strong transmission bias, thereby qualifying it as a novel selfish mitotype.

Keywords: selfish mitochondria; Caenorhabditis elegans; mutation; heteroplasm; fitness; genetic conflict; mtDNA

Introduction

Natural selection on mitochondria operates at different levels of organization where competition takes place between populations, individuals, cells, and molecules of mitochondrial DNA within cells (Rand 2001). Depending on the species, cell types, and stages of development, cells can harbor between 1 to hundreds of thousands of mitochondria, and each mitochondrion can contain multiple copies of the mitochondrial genome. Continuously occurring mtDNA mutations result in heteroplasm where novel mtDNA haplotypes are subject to genetic drift and natural selection both within and between individuals (Harrison et al. 1985; Kniec et al. 2006; Stewart and Chinnery 2015; Karavaeva et al. 2017). One consequence of the hierarchical population structure of mtDNA is that selection can favor fast-replicating mitotypes within cells that are nonetheless detrimental to the individual (Eberhard 1980; Cosmides and Tooby 1981; Barr et al. 2005). Mitotypes that enjoy a replicative or transmission advantage, sometimes bypassing or exploiting the mitochondrial quality control mechanisms of the cell, have been referred to as “selfish” as their proliferation is at the expense of individual fitness.

Intracellular selection on mtDNA can be hampered by the lack of a direct relationship between the mtDNA genotype and phenotype. In a heteroplasmic cell containing wild-type (WT henceforth) and mutant mtDNA, the consequences of reduced mitochondrial function and cellular respiration caused by mtDNA mutations may affect all mtDNA molecules, regardless of whether they contain the mutant mtDNA or not. Similarly, beneficial mtDNA mutations improving mitochondrial function may extend their fitness benefits to all mtDNA genomes in the cell. Furthermore, cells frequently respond to detrimental mtDNA mutations by increasing mtDNA copy number, a short-term solution that contributes to the proliferation of deleterious mitotypes within cells. These scenarios, referred to as the “tragedy of the cytoplasmic commons,” could lead to unchecked proliferation of deleterious mitochondrial mutations within cells with severe negative fitness consequences at the individual levels (Haig 2016). Indeed, mtDNA heteroplasmy has been implicated in mitochondrial diseases associated with senescence, infertility, and cancer in animals (Zeviani and Antozzi 1997; Wallace 1999; Keogh and Chinnery 2013; Wallace and Chalkia 2013; Greaves et al. 2014; Stewart and Chinnery 2015; Lakshmanan et al. 2018). Nuclear genomes have responded to these potentially catastrophic situations with their own adaptations, which include prevention (e.g. mtDNA repair), increasing the efficiency of selection on mtDNA variation within individuals, and compensatory mutations that ameliorate the effects of deleterious mtDNA mutations (Haig 2016; Havird et al. 2019). Small mtDNA genome size,
uniparental inheritance, mtDNA copy number bottlenecks during oogenesis, mitophagy, and apoptosis contribute to mtDNA quality control in animals (Haig 2016; Havird et al. 2019). Studying how certain mtDNA genotypes evade these mechanisms that maintain mitochondrial health and prevent the proliferation of detrimental mtDNA mutations helps us understand the coevolutionary processes of mitonuclear interactions (Estes et al. 2023). To this end, we also need to discern the types of mutations and their repertoire of selfish properties that nuclear-encoded mtDNA quality control mechanisms evolved to suppress.

*Caenorhabditis* nematodes have emerged as an important model for studying mitochondrial biology and evolution with implications for ageing, disease, and evolution (Estes et al. 2023; Onraet and Zuryn 2024). Several mtDNA deletions in *Caenorhabditis* display selfish properties in that they are strongly detrimental and, yet, persist over long periods in experimental populations and can reach high intragenomic diversity (Tsang and Lemire 2002a; Liau et al. 2007; Estes et al. 2011; Clark et al. 2012; Konrad et al. 2017; Ahier et al. 2018; Dubie et al. 2020). The first documented and most studied case of a selfish mitochondrial deletion in *Caenorhabditis elegans* is udf5, which is lacking close to 25% (11 genes) of the mitochondrial genome, including atp6, ctb-1, nd1, and nd2 (Tsang and Lemire 2002a). The udf5 deletion has persisted in laboratory populations in 60% frequency despite significant negative consequences for fitness-related traits. Additional heteroplasmic mtDNA deletions in *C. elegans* with long-term stability in laboratory populations include a 179-bp deletion in nd1, a 1-kb deletion spanning atp6, nd2, and 3 tRNA genes, and a 4.2-kb deletion spanning cox1, cox2, nd5, 16SrRNA, and 5 tRNA genes (Gitschlag et al. 2016; Meshnik et al. 2022). Collectively, these 4 mtDNA deletions include elimination of DNA from 8 out of 12 protein-coding genes in the *C. elegans* mitochondrial genome. Furthermore, several natural isolates of the congeneric *Caenorhabditis briggsae* harbor selfish mtDNA deletions in nd5 (nad5; Estes et al. 2011; Clark et al. 2012).

During a mutation accumulation (MA henceforth) experiment with obligately outcrossing *C. elegans* experimental lines bearing a fog-2 null mutation and subjected to RNAi-induced knockdown of a key mismatch repair gene msh-2 (Katju et al. 2008, 2022), a spontaneous 1,034-bp deletion originated in 1 experimental line (line 16). This deletion, designated as meuDf1, spanned the 3’ end of coxII and tRNA-θ and the 5’ end of nd4 and had reached 81.7% frequency within this line at the termination of the MA experiment. Additionally, this heteroplasmic mtDNA deletion is linked to a nonsynonymous point mutation in nd4L, which was estimated to be at 93.7% frequency (Katju et al. 2022). Here, we analyze this mtmitotype’s impact on key fitness-related traits, its population dynamics, and test for selfish behavior that may have facilitated its spread within this population.

**Materials and methods**

**Sequestering the meuDf1 mitochondria in a WT N2 background**

The original MA experiment was performed in an obligately outcrossing strain caused by a loss-of-function mutation in the fog-2 gene [fog-2(0)] that knocks out the sperm production pathway in hermaphrodites, thereby converting them to obligately outcrossing females (Katju et al. 2008, 2022; Rane et al. 2010). To directly test the phenotypic effects of the mutant mitochondria without the confounding influence of other accumulated spontaneous nuclear mutations within this experimental MA line of *C. elegans*, the mtDNA of MA line 16 was sequestered in a WT N2 nuclear background. This was accomplished by successively backcrossing females carrying the heteroplasmic meuDf1 mitotype to fog-2(0) males with a WT (non-MA) nuclear background for 10 generations during which the contribution of the nuclear DNA of the MA line was reduced by half each generation. Finally, backcrossed females with the meuDf1 mitotype were crossed with WT N2 males for another 3 generations (generations 11–13) to restore a functional fog-2 allele and convert the females to functional hermaphrodites. The use of hermaphroditic lines enables ease of manipulation when performing experiments while enabling a clean assessment of fitness effects relative to other similar mutations that have been investigated in preceding studies (Dubie et al. 2020). These functional hermaphrodites with the meuDf1 mitotype were retained to establish 3 replicate lines with the meuDf1 mitotype from MA line 16 in a WT nuclear background. These 3 lines (A–C) were cryogenically preserved at −80°C. After 13 generations of backcrossing, the proportion of the nuclear DNA from the original MA line is estimated to be 0.51 of the total nuclear DNA, or approximately 1.2 x 10⁻⁵, which virtually removes all potential nuclear mutations that may have arisen during the MA procedure. During each backcross generation, the parent worms were screened via PCR (forward primer: 5’-AGTACACAGTACGAGTGG-3’, reverse primer: 5’-AGAAGG TGGTACACCCCTATTTG-3’) to confirm the presence of the meuDf1 mitotype. The PCR products were run on a 1% agarose gel (250 mL 1× Tris-acetate EDTA; 1 g agarose; 1 μL GelRed) at 105 V for 45 min. The expected band sizes were 490 bp and ~1,500 bp for the mutant and WT, respectively.

**Phenotypic assays for 4 fitness-related traits**

Four fitness assays were performed on the 3 backcrossed lines bearing the meuDf1 mitotype. The 4 life history traits tested were productivity, survivorship to adulthood (survivorship), longevity, and developmental rate. Prior to conducting the fitness assays, frozen stocks of N2 WT (control) and the backcrossed lines (A–C) were thawed, and a parental generation was established for all lines. Four days later, the F1 generation was established by setting up 20 replicates for 4 control N2 lines (n=80) and 15 replicates for each of the 3 experimental lines (A–C; n=45). This was done by placing a single L4 hermaphrodite on a 35-mm NGM agar plate seeded with *Escherichia coli* OP50. To rid the worms of potential freezer effects, an L4 hermaphrodite was transferred for each replicate onto a new plate every 4 days for 3 generations. The F4 generation was used to conduct the assay.

For the survivorship to adulthood (or maturity) assay, 10 L1 larvae were sequestered onto a 35-mm NGM agar plate seeded with *E. coli* OP50. This was done for each replicate. Forty-eight hours later, the number of worms that were L4 or older was considered to have survived to adulthood. To assess survivorship, the number of adult worms was divided by the total number of L1 larvae initially sequestered. Survivorship values can range between 0 and 1. In *C. elegans*, development from egg to egg-laying adult typically occurs in 3.5 days. In this assay, we allowed 4 days for the sequestered worms to develop from the first larval stage (L1) to at least the fourth larval stage (L4), thereby allowing ample time for slow-developing worms to be counted as having survived to adulthood.

To measure development rate, a single L1 larva was placed on a 35-mm NGM agar plate seeded with *E. coli* OP50. To rid the worms of potential freezer effects, an L4 hermaphrodite was transferred for each replicate onto a new plate every 4 days for 3 generations. The F4 generation was used to conduct the assay.

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To measure productivity, the worms from the developmental rate assay were used. Each worm was placed on a 35-mm NGM agar plate seeded with E. coli OP50 and allowed to lay eggs. Every 24 h, the mother worm was transferred to a new plate. This was repeated each day for 8 consecutive days. After the mother worm was removed from each plate, the plate was kept in a 20°C incubator for 24 h followed by storage at 4°C. The plates were kept at 4°C for 3 weeks, after which the progeny were counted. Counting was facilitated by adding 200 μL of 0.075% solution of toluidine blue to the plate, which stains the agar but not the worms.

The same worms used for the development rate and productivity assay were used for longevity. After the last day of productivity, the worm was moved to a new NGM agar plate seeded with E. coli OP50 and checked each day until mortality. An individual was considered dead when the worm was no longer moving after being lightly prodded or there was no pharyngeal activity observed. Longevity was calculated as the number of days the worm was alive, from the L1 larval stage to death.

**Analyses of fitness data**

For each of the 4 fitness assays, data analyses were conducted using the program R. The relative fitness values for each trait in the mutant lines were calculated according to the mean absolute fitness of the N2 control lines, which were normalized to 1. The fitness measurements were determined by dividing the mean fitness of each trait observed in the mutant lines relative to that of the WT control lines.

**Competition assays**

In order to explore the fitness consequences of the meuDf1 mitotype under competitive conditions, we conducted a competition experiment. Frozen stocks of 3 backcrossed lines bearing the meuDf1 mtDNA in a WT nuclear background (A–C) and N2 WT lines were thawed. To remove freezer effects, individual worms were transferred to NGM agar seeded with E. coli OP50 and checked each day until mortality. An individual was considered dead when the worm was no longer moving after being lightly prodded or there was no pharyngeal activity observed. Longevity was calculated as the number of days the worm was alive, from the L1 larval stage to death.

**Testing for replicative advantage of the meuDf1 mitotype via an evolutionary replay experiment**

Deleterious mtDNA mutations, such as the meuDf1 mitotype can, in principle, reach high frequencies by (1) chance (genetic drift) or by (2) some form of replicative or transmission advantage within individuals. In order to determine whether the meuDf1 mitotype possessed a replicate or transmission advantage, we conducted an evolutionary replay experiment. Replay experiments using experimental evolution approaches in the laboratory serve to investigate the roles of contingency and determinism in the repeatability of evolutionary outcomes (Blount et al. 2018). To test if the meuDf1 mitotype has a competitive advantage relative to WT mtDNA within individuals, or reached a high frequency during MA due to genetic drift, we need to minimize the effects of competition between individuals. This can be accomplished by bottlenecking the C. elegans lines at a single individual in every generation. This design makes clear predictions regarding intraline selection vs genetic drift. If the frequency of meuDf1 is determined by genetic drift (chance events), there should be no significant change in the average frequency of meuDf1 across multiple independent lines that are descended from the same ancestor although the interline variation in the frequency of meuDf1 is expected to increase with the number of generations. In contrast, if the meuDf1 mitotype has a competitive advantage within individuals, the average meuDf1 frequency will increase. Lastly, within-individual selection against meuDf1 would result in decreased meuDf1 frequency across the replicate lines.

As described above under the protocol for competition assays, 6 noncompeted populations heteroplasmic for meuDf1 were maintained at high population density by chunk transfer for 60 generations, which resulted in selection against high within-individual frequency of the deletion. We screened 90 randomly selected hermaphrodites (15 random individuals from each of the 6 populations) to determine the meuDf1 heteroplasmic frequency via digital droplet PCR (ddPCR) after they had laid eggs over a 2-day period. For ddPCR, the individuals were lysed and the single worm lysates diluted 1:50 with molecular-grade water. Two Bio-Rad ddPCR fluorescent probes (FAM and HEX) were used to determine the frequency of WT to meuDf1 mtDNA in each sample. The FAM probe targeted a mtDNA segment inside the deletion and was used to estimate the concentration of WT nd4. The HEX probe targeted mtDNA outside the deletion in nd1 and was used to estimate the concentration of total mtDNA (meuDf1 + WT). Probe designs are proprietary but see Table 1 for the amplicon context of the probes. meuDf1 mtDNA frequency was calculated by subtracting the ratio of FAM to HEX concentration from 1.
Using this screening procedure, we identified a hermaphrodite with a 34% meuDf1 frequency. The offspring of this individual were sequestered to establish 15 replicate lines as part of the evolutionary replay experiment. These 15 experimental evolutionary replay lines were bottlenecked and maintained via single worm transfer each generation for 10 consecutive generations. In generations 1, 5, and 10, the mothers (n = 15) were allowed to lay eggs over a 2-day period and then lysed to determine their meuDf1 heteroplasmic frequency via ddPCR as described above.

Copy number analysis

Total mtDNA copy number was estimated using ddPCR. Randomly chosen 15 L4 C. elegans bearing the meuDf1 heteroplasm from each of the 6 backcrossed lines (NCA1, NCA2, NCB1, NCB2, NCC1, and NCC2), 90 individuals in total, were compared with 15 N2 WT individuals. A standard single worm lysis protocol was used to extract DNA from individual worms. We used a Bio-Rad FAM probe designed to target the single-copy nuclear gene, daf-3, and a Bio-Rad HEX probe targeting the ctb-1 mitochondrial gene. The ratio of mitochondrial DNA to a single-copy nuclear gene was estimated by dividing the concentration of DNA bound to the HEX probe by the concentration of DNA bound to the FAM probe. Data were analyzed using R.

Results

A deletion spanning 2 protein-coding genes was detected at a high frequency

A 1,034-bp deletion starting near the 3′ end of cxxIII and extending into nd4 (Fig. 1a) arose spontaneously in a fog-2 C. elegans line subjected to repeated msh-2 knockdown via RNAi during an MA experiment. The RNAi-induced knockdown of msh-2, a key mismatch repair gene, increased the nuclear mutation rate but was not found to directly affect the mtDNA mutation rate (Katju et al. 2022). Read depth analysis suggested 81.7% of the mtDNA in this particular MA line contained the deletion, which spanned 11.4% of cxxIII, all of the intervening tRNA-thr, and 68.2% of nd4 (Fig. 1b). In addition to this deletion designated as meuDf1, the mtDNA also contained a nonsynonymous base substitution (Leu → Pro) in the nd4L gene with an estimated heteroplasmic frequency of 93.7% based on whole-genome sequencing (WGS) analysis (Katju et al. 2022). Given the near fixation of the base substitution in nd4L and the high frequency of the deletion, we infer that the 2 mutations are linked. Furthermore, previous experiments in C. elegans have found no evidence for recombination between heteroplasmic mitochondrial deletions and base substitutions (Dubie et al. 2020). We refer to this deletion and nonsynonymous base substitution in this experimental line as the meuDf1 mitotype.

The meuDf1 mitotype causes a significant fitness reduction

Following the backcrossing regime into a WT N2 genetic background, the average heteroplasmic frequency of the meuDf1 deletion was determined to be 86.1%, which is similar to its estimated frequency from WGS analyses by Katju et al. (2022). The fitness effects of the meuDf1 mitotype were tested in a WT Bristol N2 genetic background. The backcrossed lines containing the meuDf1 mitotype were tested for 4 fitness traits: productivity, survivorship to adulthood, longevity, and developmental rate (Supplementary Table 1). These 4 traits were assayed in 3 independently backcrossed lines (A–C) harboring the meuDf1 mitotype and 4 control lines with WT mitochondria. The relative fitness of the 3 mutant lines was significantly decreased in comparison to the WT controls (Table 2 and Fig. 2). The meuDf1-bearing lines had, on average, a 63.7% reduction in productivity compared to the WT control lines (Fig. 2; Wilcoxon rank sum, z = 8.43, d.f. = 1, P < 0.0001). The productivity of meuDf1-bearing lines ranged from 97–134 progeny over the course of 8 days relative to 19–418 for the WT controls. There was no significant difference in between the 3 backcrossed deletion-bearing lines (Kruskal–Wallis rank sum, χ² = 2.50, d.f. = 2, P = 0.29). The survivorship to adulthood of meuDf1-bearing lines was 34% lower than that of the WT control lines (Fig. 2; Wilcoxon rank sum, z = 9.12, d.f. = 1, P < 0.0001). On average, only 49% of L1 larvae survived to adulthood in line C, whereas survivorship in lines A and B ranged from 71 to 75%. Survivorship of the WT control lines ranged from 81 to 100%. With respect to survivorship, line C was significantly different from lines A and B, but A and B were not significantly different from each other (Kruskal–Wallis rank sum, χ² = 9.52, d.f. = 2, P = 0.01). The average life span of meuDf1-bearing lines was significantly reduced relative to the WT controls (~11 days vs 15 days, respectively, Fig. 2; Wilcoxon rank sum, z = 3.12, d.f. = 1, P = 0.0018). This corresponds to an approximately 27% decrease in longevity. There was no significant difference in longevity between the backcrossed meuDf1-bearing lines (Kruskal–Wallis rank sum, χ² = 0.62, d.f. = 2, P = 0.73). Lastly, the meuDf1-bearing nematodes had significantly delayed development compared to the N2 control (Fig. 2; Wilcoxon rank sum, z = 7.07, d.f. = 1, P < 0.0001). The average time from egg to adult of meuDf1-bearing and WT worms was 66.3 and 47.6 h, respectively. This corresponds to the mutant-bearing worms taking, on average, 39% longer to reach adulthood. Conversely, the developmental rate of the meuDf1-bearing worms dropped by 28%.

Severe fitness decrease in meuDf1-bearing worms in competition with WT

To explore the population dynamics of meuDf1, a competition assay was performed on the 3 backcrossed lines with 2 replicates per line (6 assays), wherein meuDf1-harboring worms were directly competed with worms containing WT mtDNA. There was a sharp decrease in the frequency of meuDf1-bearing worms in all 6 competition experiments (Fig. 3a). After 7 generations, individuals with the meuDf1 mitotype were no longer detected in lines B1,
B2, C1, and C2 and only persisted in low frequency in lines A1 and A2 (Fig. 3a). The average decline in frequency of the meuDf1 mitotype was used to calculate its relative fitness (Fig. 3b). The slope of the linear regression for log(meuDf1/WT) was $-0.23$. The average relative fitness of worms carrying meuDf1 was estimated at $10^{-0.23}$, which equals 0.59, corresponding to 41% lower fitness of meuDf1-bearing worms relative to WT. In addition, 2 replicates of each backcrossed meuDf1 line were maintained for 60 generations at large population sizes as controls without competition with WT worms. In these noncompeted, large population size lines, the meuDf1 mitotype was detectable in all worms tested after 60 generations. This suggests that the extinction or decrease of mutant-bearing worms in the competed populations was due to competitive disadvantage and loss of meuDf1-harboring worms and not due to loss of the meuDf1 heteroplasmy within worms (Fig. 3b).

**The meuDf1 mitotype exhibits selfish drive**

We conducted several experiments to test whether genetic drift or selfish drive contributed to the increased heteroplasmic frequency of meuDf1 within this experimental *C. elegans* line. We used the offspring of an individual hermaphrodite harboring...
meuDf1 at a 34% frequency as the parental generation to establish 15 replicate lines. In this follow-up experiment, we relaxed interindividual selection by subjecting these 15 replicate meuDf1 lines to single individual bottlenecks in every generation for 10 consecutive generations as per an earlier protocol (Dubie et al. 2020). If the intraindividual dynamics are dominated by genetic drift, there should be no significant change in the average meuDf1 frequency; however, the variance in heteroplasmy frequency between lines is expected to increase with time. If there is intraindividual selection against the meuDf1 mitotype, the average frequency is expected to decline. Conversely, if the meuDf1 has a replicative advantage, its average frequency is expected to increase. Over the course of 10 generations, the frequency of meuDf1 more than doubled, rising to an average of 77% (Fig. 4; Supplementary Table 2). The average frequency of meuDf1 in the first generation of the bottlenecked lines was similar to the parent

**Table 2.** Fitness of meuDf1-bearing mtDNA in a WT nuclear background relative to control worms of the Bristol N2 laboratory strain harboring WT mtDNA.

<table>
<thead>
<tr>
<th>Fitness-related trait</th>
<th>Productivity</th>
<th>Survivorship to adulthood</th>
<th>Longevity (days)</th>
<th>Developmental time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>z̅ N2 control</td>
<td>307.95</td>
<td>0.99</td>
<td>14.81</td>
<td>47.57</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 16</td>
<td>111.67</td>
<td>0.65</td>
<td>10.98</td>
<td>66.25</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 1</td>
<td>295.79</td>
<td>0.995</td>
<td>16.10</td>
<td>47.26</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 2</td>
<td>293.00</td>
<td>0.990</td>
<td>14.45</td>
<td>47.79</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 3</td>
<td>326.50</td>
<td>0.990</td>
<td>15.35</td>
<td>47.33</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 4</td>
<td>317.47</td>
<td>0.985</td>
<td>13.35</td>
<td>47.89</td>
</tr>
<tr>
<td>z̅ N2 control</td>
<td>104.57</td>
<td>0.710</td>
<td>10.83</td>
<td>67.00</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 16</td>
<td>96.80</td>
<td>0.760</td>
<td>11.20</td>
<td>64.92</td>
</tr>
<tr>
<td>z̅ meuDf1-bearing line 16</td>
<td>133.64</td>
<td>0.490</td>
<td>10.83</td>
<td>67.00</td>
</tr>
</tbody>
</table>

Twenty replicates each of 4 N2 control lines (n = 80) and 15 replicates each for 3 meuDf1-bearing lines (n = 45) were assayed. Estimates of the mean phenotype for 4 fitness traits are provided for the WT N2 control (z̅ N2 control; n = 80) and the meuDf1-bearing lines (z̅ meuDf1-bearing line 16; n = 45). Mean fitness values across n = 20 and 15 replicates for individual control and experimental lines, respectively, are also provided (4 N2 control and 3 meuDf1-bearing lines).

**Fig. 2.** Relative trait means ± SD of the meuDf1-bearing replicate lines vs the WT N2 control. Phenotypic assays were conducted for 4 life history traits, namely (1) productivity, (2) survivorship to adulthood, (3) longevity, and (4) developmental rate. Mean fitness values for each of the 4 traits were measured across 3 backcrossed MA16 lines, each with 15 replicates where possible (n = 45) and 4 N2 control lines with 20 replicates each (n = 80). For simplicity, the mean relative fitness value for each of the 4 traits in the WT N2 control was scaled to a value of 1. There was a significant decrease in the mean trait value in all the meuDf1-bearing lines relative to the WT N2 lines. **P ≤ 0.01 and ****P ≤ 0.0001.
Copy number was significantly elevated in mutant lines

The relative copy number of mtDNA has frequently been observed to increase in the presence of a high-frequency deleterious mtDNA heteroplasm (Tsang and Lemire 2002a; Gitschlag et al. 2016; Lin et al. 2016; Dubie et al. 2020). Furthermore, mtDNA copy number increase has been suggested as a driving force in the proliferation of deleterious mtDNA heteroplasmies (Tam et al. 2013; Gitschlag et al. 2016). The relative mtDNA copy number was estimated for 30 individuals with the meuDf1 backcrossed into an N2 background and compared with mtDNA copy number in 15 N2 individuals with WT mtDNA (Fig. 5 and Supplementary Table 3). The median relative mtDNA copy number in the meuDf1 and WT mtDNA individuals was 62 and 46, respectively, thereby representing an increase of 32% in the former (Mann-Whitney test: U = 125, P = 0.004).

Discussion

The mitochondrial and nuclear genomes have been coevolving for approximately 1.5 billion years, resulting in the finely tuned expression of thousands of nuclear genes and several remnant mitochondrial genes that are essential for mitochondrial function (Lang et al. 1999). However, the high mutation rate of most animal mitochondrial genomes constantly creates heteroplasm with the introduction of potentially harmful mtDNA mutations. Because the heredity of mtDNA is non-Mendelian, these harmful mtDNA mutations can sometimes proliferate if their deleterious fitness effects are limited to the sex that does not pass on mtDNA to future generations, or by a transmission or replication bias that favors the detrimental mutation (Eberhard 1980; Cosimides and Tooby 1981). Despite the evolution of a diversity of nuclear-encoded mechanisms to limit the proliferation of selfish or cheater mitochondrial genomes, harmful mtDNA mutations sometimes manage to reach high frequency under certain conditions (Haig 2016; Havird et al. 2019). These selfish mtDNA mutations provide us with an opportunity to probe the role played by genetic conflict in the evolution of the 2 respective genomes.

To qualify as a selfish mitochondrial mutation, a variant must fulfill 2 criteria: (1) it should have a neutral or deleterious fitness effect and (2) a transmission advantage. In the case of the meuDf1 mitotype, we have herein demonstrated that the deleterious effects are large by any standard, including reduced productivity, a slower rate of development, lower survivorship to adulthood, and reduced longevity. Furthermore, composite fitness was reduced by 41% in individuals harboring this deletion when competed with individuals containing WT mtDNA. With such a large fitness disadvantage, a transmission advantage might be difficult to discern unless the efficiency of individual selection for reduced heteroplasm frequency is limited to better reveal the within-individual dynamics. This was accomplished by single individual bottlenecks in every generation. Under these conditions, the average frequency of meuDf1 increased rapidly in frequency from 0.34 to 0.77 in a mere 10 generations. The severe fitness cost combined with an increase in the frequency of the meuDf1 mitotype when selection between individuals is limited serve as the hallmarks of a selfish mtDNA mitotype.

The identification of heteroplasmic mtDNA deletions in Caenorhabditis nematodes provide an opportunity to delineate their effects on organismal fitness and oxidative phosphorylation with their ensuing consequences for mtDNA copy number dynamics, mitonuclear coevolutionary interactions, and critical threshold ranges for the onset of disease phenotypes. As with any mutation, the fitness consequences of these heteroplasmic mtDNA deletions are likely to be context dependent and influenced by several factors including (1) the size of the deletion and, hence, the number of genes affected, (2) the mutational effect (frameshift vs in-frame), and (3) the heteroplasmic frequency, among others. However, this comes with the caveat that the
influence of these factors on organismal fitness are confounded by differences in laboratory (rearing and environmental conditions), measures of different life history or fitness traits, and the use of different protocols. The uAf5 mtDNA deletion was identified in a laboratory population of C. elegans following an ethyl methanesulfonate (EMS) mutagenesis screen (Tsang and Lemire 2002b). This 3.04-kb deletion resulted in the partial or complete deletion of 4 protein-coding mtDNA genes (nd1, atp6, nd2, and ctb-1) and 7 tRNA genes and ranged in 20–80% frequency within laboratory lines with an average heteroplasmic frequency of ~60% (Tsang and Lemire 2002b; Liau et al. 2007). A preceding study determined that the egg-laying frequency, longevity, and defecation frequency of uAf5-bearing worms were negatively correlated with uAf5 frequency (Liau et al. 2007). Our employment of a different set of key fitness traits and composite fitness inferred from competition experiments with uAf5-bearing worms preclude a direct comparison. However, our measure of productivity, a key fitness trait that is extremely sensitive to mutation load and pressure (Katju et al. 2015, 2018) permits comparisons to the heteroplasmic And5 deletion in wild isolates of C. briggsae (Estes

Fig. 4. a) Schematic of the evolutionary replay experiment to test if the meuDf1 mitotype has a competitive advantage relative to WT mtDNA within individuals. Ninety adult hermaphrodites were randomly selected from experimental populations maintained at large sizes and screened for meuDf1 frequency via ddPCR (see Materials and methods for details). The offspring of a single worm with the meuDf1 mitotype in 34% frequency were used to establish 15 experimental evolution lines, which were subjected to single individual bottlenecks each generation for 10 consecutive generations. The frequency of the meuDf1 mitotype was measured by ddPCR in generations 1, 5, and 10. Created with BioRender.com. b) The change in heteroplasmic frequency of meuDf1 during 10 generations of bottlenecking via single progeny descent in 15 replicate lines. The lines were established by 15 offspring of a single individual with 34% heteroplasmic frequency of meuDf1, indicated by a dashed line. The average heteroplasmic frequency across the 15 lines increased to 77%. There was a significant increase in the average frequency of meuDf1 from generations 1 to 5 (34% to 56%, respectively; paired t-test: t = 5.82, P < 0.0001). Similarly, the average frequency of meuDf1 increased significantly from generations 5 to 10 (56% to 77%, respectively; paired t-test: t = 6.06, P < 0.0001).
The 2 protein-coding genes affected by deletion in the meuDf1 mtmitotype are coxII and nd4 (Fig. 1). nd4 codes for a hydrophobic inner subunit of ETC complex I and is involved in proton transfer (Hirst 2009; Efremov et al. 2010). CoxII codes for one of the catalytic cores of ETC complex IV and is important for complex assembly (Capaldi 1990; Meunier and Tannman 2002). Studies have also confirmed that disruption to ETC complexes I and IV results in an increased production of free radicals, causing more oxidative stress on the mitochondria (Grad and Lemire 2004; Lenaz et al. 2004; Distelmaier et al. 2009). Hence, meuDf1 likely leads to the misfolding of multiple subunits of the ETC complexes with the potential for engendering complex deficiencies. However, a determination of the direct impact(s) of the meuDf1 mutation on mitochondrial function requires further study.

Mitochondrial DNA deletions have been associated with replication or transmission advantage in several systems, including Saccharomyces, Drosophila, and Caenorhabditis (Volz-Lingenhöhl et al. 1992; MacAlpine et al. 2001; Taylor et al. 2002; Tsang and Lemire 2002a, 2002b; Clark et al. 2012; Jasmin and Zeyl 2014; Dubie et al. 2020). Within Caenorhabditis, natural isolates of C. briggsae have been found with a deletion in nd5 (nad5'), which increases in intracellular frequency when individual selection is relaxed, a classic symptom of selfish mtDNA and a conflict between different levels of selection (Clark et al. 2012). Furthermore, the transmission advantage of nad5A was frequency dependent within individuals, greatest when heteroplasmy levels of Δnad5 were low and diminishing with increased frequency of the deletion (Clark et al. 2012). The classic example of a selfish mtDNA deletion in C. elegans is uad5', which affects a quarter of the 12 protein-coding genes in the C. elegans mitochondrial genome, namely atp6, ctb-1, nd1, and nd2 (Tsang and Lemire 2002a, 2002b; Clark et al. 2012). Within Caenorhabditis, natural isolates of C. briggsae have been found with a deletion in nd5 (nad5'), which increases in intracellular frequency when individual selection is relaxed, a classic symptom of selfish mtDNA and a conflict between different levels of selection (Clark et al. 2012).

Furthermore, the transmission advantage of nad5A was frequency dependent within individuals, greatest when heteroplasmy levels of Δnad5 were low and diminishing with increased frequency of the deletion (Clark et al. 2012). The classic example of a selfish mtDNA deletion in C. elegans is uad5', which affects a quarter of the 12 protein-coding genes in the C. elegans mitochondrial genome, namely atp6, ctb-1, nd1, and nd2 (Tsang and Lemire 2002a, 2002b; Clark et al. 2012). Within Caenorhabditis, natural isolates of C. briggsae have been found with a deletion in nd5 (nad5'), which increases in intracellular frequency when individual selection is relaxed, a classic symptom of selfish mtDNA and a conflict between different levels of selection (Clark et al. 2012). Furthermore, the transmission advantage of nad5A was frequency dependent within individuals, greatest when heteroplasmy levels of Δnad5 were low and diminishing with increased frequency of the deletion (Clark et al. 2012).
mitochondrial mutations (Gitschlag et al. 2016, 2020; Filograna et al. 2019; Dubie et al. 2020). This is thought to be a compensatory mechanism aimed at sustaining mitochondrial oxidative phosphorylation, coordinated by the nuclear genome (Gitschlag et al. 2016). Ironically, this observed compensatory increase in mtDNA has been hypothesized to aid in the proliferation of mtDNA mutations (Tam et al. 2013; Gitschlag et al. 2016). It has been shown that ATFS-1, which accumulates in dysfunctional mitochondria, promotes the binding of the mtDNA polymerase POLG to mtDNA, which results in preferential replication of AmtDNA (Yang et al. 2022). Overreplication of mutant mtDNA in C. elegans should also apply to other classes of mtDNA mutations (base substitutions and small indels) resulting in nonsense mutations, frameshifts, and certain nonsynonymous mutations. However, there are no reported cases of base substitutions or frameshift mutations, which on their own have selfish mtDNA properties in C. elegans. This may stem from an ascertainment bias that favors easy to observe molecular changes such as large deletions or duplications. The deletion of critical genes is striking and demands an explanation whereas base substitutions might not elicit the same kind of attention. Some of the deletion heteroplasmies in C. elegans do contain additional base substitutions, which may contribute to their selfish properties. In the case reported here, these meudF1 molecules also harbor a nonsynonymous mutation in nd4L, and a previously reported mitochondrial ctb-1 deletion is linked to mutations in nd5 (Dubie et al. 2020).

The high mutation rate in mtDNA relative to the nucleus may predispose mitochondrial genomes to MA, and it is possible that a subset of deleterious mtDNA mutations are not just passively accumulating by genetic drift but are increasing in frequency by exploiting the nuclear regulation of mtDNA replication and bypassing mitochondrial quality control. The origin and proliferation of selfish organelles can lead to population extinction if left unabated but also sets the stage for compensatory evolution within individuals, and they are unlikely to survive in the wild considering their large fitness costs. Yet, a deletion in Dobzhansky–Muller incompatibilities (BDMIs) (Sloan 2020). Large-scale mtDNA deletions cannot reach fixation within individuals, and they are unlikely to survive in the wild considering their large fitness costs. Yet, a deletion in nd5 has been found in natural isolates of C. briggsae (Howe and Denver 2008; Clark et al. 2012). However, these deletions provide us with an excellent opportunity to probe how deleterious mtDNA mutations can reach high frequency within individuals, the various adaptations to contain selfish mitochondria, and the evolutionary dynamics of mitonuclear genetic conflict.

Data availability

All data including fitness data have been made available in the supplementary material file. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables. Supplemental material available at G3 online.

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Conflicts of interest

The authors declare no conflicts of interest.

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